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In situ synthesis of exopolysaccharides by *Leuconostoc* spp. and *Weissella* spp. and their rheological impacts in fava bean flour

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Abbreviations:

ANF, anti-nutritional factors; LAB, lactic acid bacteria; EPS, exopolysaccharides; TTA, total titratable acidity; HPAEC-PAD, high performance anion exchange chromatography with pulse amperometric detection; WHC, water-holding capacity; RFO, raffinose family oligosaccharides; FQ, fermentation quotient.

ABSTRACT

Fava bean flour is regarded as a potential plant-based protein source, but the addition of it at high concentration is restricted by its poor texture-improving ability and by anti-nutritional factors (ANF). Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) are regarded as good texture modifiers. In this study, fava bean flour was fermented with *Leuconostoc* spp. and *Weissella* spp. with or without sucrose addition, in order to evaluate their potential in EPS production. The contents of free sugars, organic acids, mannitol and EPS in all fermented fava bean doughs were measured. Rheological properties of sucrose-enriched doughs, including viscosity flow curves, hysteresis loop and dynamic oscillatory sweep curves, were measured after fermentation. As one of the ANF, the degradation of raffinose family oligosaccharides (RFO) was also studied by analysing RFO profiles of different doughs. Quantification of EPS revealed the potential of *Leuconostoc pseudomesenteroides* DSM 20193 in EPS production, and the rheological analysis showed that the polymers produced by this strain has the highest texturing capability. Furthermore, the viscous fava bean doughs containing plant proteins and synthesized *in situ* EPS may have a potential application in the food industry and fulfill consumers' increasing demands for "clean labels" and plant-originated food materials.

Keywords: exopolysaccharides; lactic acid bacteria; fava bean; fermentation; texture modification

1. Introduction

Fava bean (*Vicia faba* L.) is a widely grown crop utilized for food and animal feed in many countries (Duc, 1997). Fava bean seeds are very nutritious due to their high contents of proteins, vitamins, minerals and dietary fibers (Jezierny et al., 2010). Also, fava bean is a rich source of non-nutrient secondary metabolites, such as phenols and flavonoids, which have antioxidant activities and positive effects on human health (Yuwei et al., 2014). Moreover, fava bean consumption shows a positive effect on decreasing LDL-cholesterol levels in plasma and on Parkinson's disease motor symptoms due to the presence of L-3, 4-dihydroxyphenylalanine (L-DOPA) (Frühbeck et al., 1997; Ramírez-Moreno et al., 2015). Although it possesses these beneficial properties, fava bean still remains underutilized, particularly for human consumption in European countries where meat and dairy products are the major protein sources in the diet (Westhoek et al., 2014). One reason for the limited utilization of fava bean is the anti-nutritional factors (ANF) such as raffinose family oligosaccharides (RFO), tannins, protease inhibitors, alkaloids and lectins (Liener, 1990). They are contained in raw fava bean seeds and are known to exert anti-nutritional functions, reducing their digestibility and even leading to some pathological reactions (Gupta, 1987).

In some studies, the decrease of ANF in fava bean has been addressed with different processing methods, such as soaking, air classification, extrusion, heat treatment and fermentation (Alonso et al., 2000; Coda et al., 2015; Luo and Xie, 2013). In particular, fermentation with lactic acid bacteria (LAB) has shown to be effective in reducing ANF and at the same time to improve the nutritional values of fava bean (Coda et al., 2015). Another property of LAB is their ability to synthesize exopolysaccharides (EPS), which has been widely exploited for milk and cereal fermentations (Galle et al., 2010; Galle et al., 2012; Gentès et al., 2011; Purwandari et al., 2007) and could be exploited for legume fermentation as well. EPS are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives (Welman and Maddox, 2003). According to the composition, EPS can be classified as homopolysaccharides, which are composed of only one type

of sugar unit (e.g., glucose), and heteropolysaccharides, which are composed of two or more types of sugar units (e.g., galactose and rhamnose). Normally, EPS are secreted into their surroundings during microbial growth and are not attached permanently to the cell surface (Laws et al., 2001), protecting microbial cells from environmental stresses, e.g., nisin, starvation, membrane stress and low pH (Kim et al., 2000; Looijesteijn et al., 2001; Schwab and Gänzle, 2006).

Microbial EPS have been regarded as alternatives for plant polysaccharides that are commonly used as thickening, stabilizing, texturizing and gelling agents in the food industry (Galle and Arendt, 2014). In particular, EPS produced by LAB have been applied to improve the rheological, textural and sensory properties of yogurt (Folkenberg et al., 2005; Welman and Maddox, 2003). It was reported that EPS from LAB increased the stay time of milk products in the mouth, imparting an enhanced perception of taste (Duboc and Mollet, 2001). Some health-promoting effects of EPS, e.g., prebiotic, immune-potentiating and cholesterol-lowering activity, were also reported (Chabot et al., 2001; Guo et al., 2013; Hosono et al., 1997; Nakajima et al., 1992). Furthermore, EPS were produced during food fermentation by LAB and hence were not manually added to food products, thus meeting consumers' demands for decreased usage of food additives. To the best of our knowledge, there is still very limited information on the rheological properties of legumes containing EPS synthesized *in situ* (Hickisch et al., 2016). The presence of these EPS might contribute to the exploitation of fava bean potential, particularly as a candidate for meat substitution (Multari et al., 2015).

The objective of this study was to evaluate the potential of different LAB starters in EPS production and texture modification in fava bean flour. The biochemical and rheological properties of fava bean doughs before and after fermentation were assessed and compared. Due to the nutritional importance of RFO, the effect of LAB fermentation on RFO load in fava bean flour was also investigated. Based on the EPS contents and rheological properties of the fermented fava bean doughs, the most promising LAB strain for EPS production could be identified.

2. Materials and methods

2.1 LAB and raw material

Six LAB strains belonging to *Leuconostoc* spp. and *Weissella* spp., previously selected according to their EPS-producing abilities on sucrose-supplemented MRS agar, were used for fava bean flour fermentation. In detail, *Leuconostoc pseudomesenteroides* DSM 20193, *Leuconostoc mesenteroides subsp. mesenteroides* DSM 20240, *Leuconostoc citreum* DSM 5577 and *Weissella cibaria* DSM 15878 were purchased from Leibniz Institute DSMZ (Braunschweig, Germany). *Weissella cibaria* Sj 1b and *Weissella confusa* Sj5-4, previously isolated from onion powder, were obtained from the culture collection of the Division of Food Hygiene and Environmental Health, University of Helsinki. *Lactobacillus plantarum* DPPMAB24W, which belongs to the culture collection of the Department of Soil, Plant and Food Sciences, University of Bari, was used as an EPS-negative control. All LAB strains were routinely propagated in MRS broth at 30 ºC (Oxoid, Basingstoke, Hampshire, England).

Fava bean (*Vicia faba* major, harvest year 2014) flour was purchased from CerealVeneta (San Martino di Lupari, PD, Italy). The composition of the flour was: protein (35.7%), carbohydrates (49.3%), lipids (1.6%), moisture (9.5%) and ash (3.9%), calculating on dry matter base.

2.2 Dough fermentation

Doughs were prepared according to the recipe reported in Table 1. Forty grams of fava bean flour were mixed with 60 mL of distilled water (control doughs). In order to enable EPS formation, 25% (w/w) of fava bean flour was replaced by sucrose and mixed with 60 mL of distilled water (sucrose-enriched doughs). Microbial cells, propagated in MRS broth supplemented with 2% (w/v) sucrose overnight, were sedimented by centrifugation (10,000 × *g* for 10 min) and washed with 50 mM sodium phosphate buffer, pH 7.0. Then, cells were re-suspended in distilled water to inoculate doughs at a cell density range of ca. 5.0 – 6.0 log cfu/g. Fermentation was carried out at 30 ºC for 24 h.

2.3 LAB cell density, pH and total titratable acidity (TTA)

LAB cell density was analyzed before and after fermentation. Dough samples (10 g) were homogenized with 90 mL of sterile saline solution in a Stomacher 400 lab blender (Seward Medical, London). Serial dilutions were made and the diluted bacterial suspensions were plated on MRS agar (Oxoid, Basingstoke, Hampshire, England). After 48 h of incubation at 30 ºC, bacterial colonies were counted. The pH values were determined by a pH meter (Model HI 99161, Hanna Instruments, Woonsocket, RI, USA) with a food penetration probe. TTA was measured by a Mettler Toledo EasyPlus Titrator (Schott, Germany). Ten grams of sample in 100 mL of distilled water were titrated with 0.1 M NaOH to a final pH of 8.5. TTA was expressed as the volume of NaOH used (mL).

2.4 Analysis of sugars, mannitol and organic acids

One hundred milligrams of freeze-dried samples were mixed with 5.0 mL of Milli-Q water and vortexed for 5 min to allow the complete dissolution of free sugars, mannitol and organic acids. Then, the suspensions were boiled for 5 min to inactivate enzymes and microbes. After cooling, samples (400 µL) were filtered using Amicon Ultra-0.5 centrifugal filter units (Millipore, Billerica, MA) at 12,000 × g for 10 min to remove polymeric molecules (above 10 kDa). Before further analysis, samples were diluted with Milli-Q water.

2.4.1 Sugars

Mono-, di- and oligo-saccharides in fava bean doughs were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) system. Sugars were separated in a CarboPac PA1 column (250 × 4 mm i.d., Dionex, Sunnyvale, CA) with a flow rate of 1 mL/min and were detected by a Waters 2465 pulsed amperometric detector (Waters, USA). The gradient elution started from 2 mM NaOH (3 min), then to 60 mM NaOH (32 min), and finally to 200 mM (8 min) followed by washing and regeneration. The injection volume was 10 µL. Glucose (Merck, Germany), fructose (Merck), galactose (Sigma-Aldrich), sucrose (Merck), melibiose (Sigma-Aldrich), raffinose (Sigma-Aldrich), stachyose (Sigma-Aldrich) and verbascose (Megazyme, Ireland) were

used as standards and 2-deoxy-D-galactose (Sigma-Aldrich) was used as the internal standard for quantification.

In order to confirm the function of microbial enzymes and endogenous α-galactosidase in fava bean flour on RFO degradation, both native and autoclaved fava bean flour were used for control dough preparation and were inoculated with the same starter, as shown in Table 1. After 24 h fermentation, doughs made with both native and autoclaved flour were treated by the same method according to Section 2.4. Then, the sugar profiles of these doughs were analyzed using the same method as stated above.

2.4.2 Mannitol

Mannitol was quantified by the HPAEC-PAD system equipped with a CarboPac MA-1 analytical column (4 × 250 mm i.d., Dionex), a DECADE detector (Antec Leyden, The Netherlands), a Waters 717 Autosampler and two Waters 515 pumps. The gradient elution started from 40 mM NaOH (25 min), then to 620 mM (10 min), and finally to 40 mM. The flow rate was 0.4 mL/min and the injection volume was 5 μL. Mannitol (Sigma-Aldrich) was used as the standard for quantification.

2.4.3 Organic acids

Organic acids were separated by an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, USA) and were detected by a Waters 2487 Dual λ Absorbance Detector (operating at 210 nm). Sulfuric acid (10 mM) was used as the mobile phase with a flow rate of 0.6 mL/min. The injection volume was 20 μL. Citric acid (Sigma-Aldrich), L-lactic acid (Sigma-Aldrich) and acetic acid (Merck) were used as standards for quantification.

2.5 EPS analysis

2.5.1 Dextran

157 The amount of low-branched dextran was analyzed by an enzyme-assisted method using a mixture
158 of dextranase (Sigma-Aldrich) and α-glucosidase (Megazyme, Ireland) according to a method
159 reported earlier (Katina et al., 2009).

160 **2.5.2 Glucan**

161 The total glucan content was quantified by calculating the content of released glucose after sulfuric
162 acid hydrolysis. Freeze-dried samples (100 mg) were washed twice with 50% ethanol to remove
163 monosaccharides and oligosaccharides. Then, 3 mL (30 U) of thermostable α-amylase (Megazyme)
164 in 100 mM (pH 5.0) sodium acetate buffer were added. Samples were then incubated in a boiling
165 water bath for 16 min to degrade starch. After cooling, 100 μL (20 U) of amyloglucosidase
166 (Megazyme) was added, and samples were further incubated at 50 °C for 30 min, followed by
167 precipitation with 10 mL of ethanol (99.6%). Then, samples were kept at 4 °C overnight to allow the
168 complete precipitation of soluble EPS. After centrifugation (10,000 × *g*, 15 min), the supernatant was
169 removed and 1 mL of 1.0 M sulfuric acid was added to hydrolyze glucan at 100 °C for 2 h. Finally,
170 samples were neutralized with 2 mL of Na_2CO_3 (1.0 M) and diluted for sugar analysis as described
171 in 2.4.1. Glucose was treated under the same conditions and was used as the standard for
172 quantification. The produced glucan contents were calculated by subtracting the glucan contents in
173 unfermented samples.

174 **2.6 Rheological analysis**

175 **2.6.1 Viscosity**

176 Before and after fermentation, samples (60 g) were taken and the viscosity values were measured at
177 20 ºC with a RheolabQC rheometer (Anton Paar, Austria) at different shear rates from 2 to 100 1/s
178 (up and down sweeps). To calculate the specific viscosity increase, the viscosity values at the shear
179 rate of 100 1/s were used.

180 **2.6.2 Water-holding capacity (WHC)**

Centrifuge tubes containing fermented samples were first weighed and then centrifuged at 8,000 × g for 10 min. The liquid that appeared after centrifugation was removed with a needle connected to a syringe. Then, samples were weighed again. WHC was expressed in percentage as the ratio between the weight after liquid removal and the initial weight. Measurements were carried out in triplicate.

2.6.3 Hysteresis loop

The hysteresis loop curve was generated by measuring shear stresses as a function of shear rates from 2 to 100 1/s (up and down sweeps) by a HAAKE RheoStress rheometer (RS 50, HAAKE Rheometer, Karlsruhe, Germany). Samples were left for 5 min to equilibrate before measurement. The hysteresis loop area between the upward and downward flow curves was calculated using the RheoWin Pro software.

2.6.4 Dynamic oscillatory sweep

The dynamic moduli (G', G") were recorded as a function of frequency from 0.1 to 10 Hz by a HAAKE RheoStress rheometer at 20 ºC. Measurements were conducted in triplicate after sample equilibration.

2.7 Statistical analysis

The numerical results of microbial, chemical and rheological analysis in this study are averages of three independent replicates. Data were analyzed by one-way analysis of variance (ANOVA) using Origin 8.6 (OriginLab Inc., USA). The means comparisons were determined by Tukey's test ($P < 0.05$).

3. Results and discussion

3.1 LAB growth and acidification

After 24 h fermentation, the LAB cell density ranged from 8.6 to 9.8 log cfu/g, increasing between 2.8 to 4.0 log cycles (Table 2). In detail, the highest cell density increase (Δ log) was observed in

5577_S, and the lowest was in 20240_S. Overall, the addition of sucrose did not significantly affect the final cell density and the cell density increase as already reported in a similar study (Kajala et al., 2015).

Before fermentation, the pH of the fava bean doughs was ca. 6.6; it dropped 1.6 to 2.3 units after fermentation, reaching values in the range from 4.3 to 5.0 (Table 2). Fermented with the same starter, sucrose-enriched doughs showed slightly lower pH compared with control doughs. According to Table 2, TTA values varied from 10.7 to 19.8 mL, with the highest value in 5577_S and the lowest value in Sj5-4_S. Generally, doughs fermented by *Weissella* spp. were less acidic (higher pH and lower TTA) than doughs fermented by *Leuconostoc* spp., which was also reported earlier (Katina et al., 2009).

Citric acid was found in fava bean doughs, before and after fermentation, with some differences among samples (Table 2). In the two doughs fermented by either *Ln. citreum* DSM 5577 (5577_CT and 5577_S) or *W. confusa* Sj5-4 (Sj5-4_CT and Sj5-4_S), citric acid was no longer detectable, indicating its involvement in microbial metabolism. In the other cases, no significant differences were found in citric acid concentration between the control and sucrose-enriched doughs fermented with the same starter. Lactic acid concentration ranged from 9.50 to 21.97 mmol/100 g dough, with the highest concentration in 20193_CT and the lowest in 20240_S. With the addition of sucrose, lactic acid concentration decreased to differing extents. In the case of sucrose-enriched doughs fermented by *Leuconostoc* spp. (20193_S, 20240_S, 5577_S), more acetic acid was formed after sucrose addition, particularly in 5577_S. By contrast, the acetic acid concentration in sucrose-enriched doughs fermented by *Weissella* spp. (15878_S, Sj 1b_S, Sj5-4_S) was less affected by sucrose addition, which was also mirrored by their less varied fermentation quotient (FQ) compared with *Leuconostoc* spp. (Table 2). This difference between the heterofermentative species *Leuconostoc* and *Weissella* might be due to different metabolic pathways of fructose liberated from hydrolysis of the added sucrose by the activity of a glucansucrase. It was previously observed that the mannitol dehydrogenase of

229 *Leuconostoc* spp. could reduce *in vivo* fructose to mannitol, contributing to the formation of acetic
230 acid (Wisselink et al., 2002). On the contrary, *Weissella* spp. ferment fructose but do not reduce it to
231 mannitol, leading to low acetic acid production (Galle et al., 2010; Kajala et al., 2015).

232 **3.2 Sugar analysis**

233 Before fermentation, sugars in control and sucrose-enriched doughs were analyzed, and the existence
234 of endogenous sucrose was revealed (Table 3). After fermentation, sucrose was utilized by LAB to
235 different extents. In all control doughs without added sucrose, endogenous sucrose was no longer
236 detected, while in two sucrose-enriched doughs, 15878_S and Sj 1b_S, almost half of the total sucrose
237 remained. In other sucrose-enriched doughs, sucrose was depleted or only a small amount (0.30%)
238 was detected after fermentation. Theoretically, sucrose was first utilized for microbial growth during
239 the exponential phase, then for EPS production as slime EPS were produced during the stationary
240 phase (Plante and Shriver, 1998). Through the activity of glucansucrase on sucrose, glucan was
241 formed, liberating fructose. This was further confirmed by the residual fructose in all sucrose-
242 enriched doughs, ranging from 1.76% to 5.37% (Table 3).

243 RFO (raffinose, stachyose and verbascose) were detected in control doughs before fermentation as
244 these oligosaccharides are widely distributed in the leguminous family (Aguilera et al., 2009). After
245 fermentation, the content of RFO decreased in each case, as expected (Table 3). In detail, raffinose
246 was no longer detected while stachyose and verbascose were still detected in control doughs with
247 varying contents, from 0.24% to 0.85% and from 0.31% to 0.99% respectively. Stachyose and
248 verbascose were not detected in sucrose-enriched doughs due to the reduced flour content in those
249 doughs. Galactose, which is a degradation product of RFO by α-galactosidase, was also detected, in
250 agreement with our previous study on fava bean fermentation (Xu et al., unpublished results). In order
251 to assess the role of microbial enzymes and native α-galactosidase of fava bean seeds (Dey and
252 Pridham, 1969) in RFO degradation, the fava bean flour used in this study was autoclaved to
253 inactivate the endogenous enzymes, and used to prepare doughs under the same conditions as native

flour. The influence of microbial and endogenous enzymes on RFO degradation was evaluated by comparing the sugar profiles of control doughs made with native or autoclaved flour with the same starter (Fig. 1). According to Fig. 1, the sugar profiles changed after autoclaving. In detail, galactose was not detected after autoclaving, and the contents of RFO in doughs prepared with autoclaved flour were generally higher than those in doughs prepared with native flour, and were comparable to unfermented flour, with the single exception of the dough fermented by *Ln. pseudomesenteroides* DSM 20193. In this dough, raffinose and stachyose disappeared, indicating a presumptive α-galactosidase activity of *Ln. pseudomesenteroides* DSM 20193, as α-galactosidase in fava bean flour was inactivated by autoclaving. The produced galactose might have been consumed during fermentation since *Ln. pseudomesenteroides* DSM 20193 was reported to be able to ferment galactose and raffinose (Farrow et al., 1989). In a departure from our previous work (Xu et al., unpublished results), other degradation products, such as melibiose, manninotriose and manninotetraose, were not detected in this study due to the absence of microbial levansucrase. This was further confirmed by screening all LAB strains on MRS agar containing 2% (w/v) raffinose, on which no EPS formation was observed (data not shown). Notwithstanding the major role of fava bean endogenous α-galactosidase in RFO degradation, the content of residual stachyose and verbascose in doughs fermented without sucrose addition still varied, indicating the effect of different microorganisms on activation of the endogenous enzymatic activities of the flour.

3.3 EPS and mannitol

The addition of sucrose strongly induced dextran and glucan production when comparing the dextran and glucan contents in control and sucrose-enriched doughs fermented with the same starter (Table 4). Glucan contents were higher than dextran contents in all cases, as low- and high-branched dextrans were both included in glucans. Dextran content varied from 0.11% to 0.74% in control doughs and from 1.86% to 3.67% in sucrose-enriched doughs. When only endogenous sucrose was available, *W. cibaria* Sj 1b produced the highest amount of dextran compared with other starters. However, with

279 the addition of more sucrose, this strain produced the lowest amount of dextran, indicating a possible
280 repression to glucansucrase of *W. cibaria* Sj 1b due to the high substrate concentration (Shukla and
281 Goyal, 2011). Glucan content ranged from 0.32% to 0.82% in control doughs and from 2.57% to 4.33%
282 in sucrose-enriched doughs, with the highest content in 20240_S and the lowest in Sj5-4_S. In the
283 presence of the same amount of sucrose, *Leuconostoc* spp. produced more glucan than did *Weissella*
284 spp. in sucrose-enriched doughs (Table 4). Additionally, smaller differences were found between
285 dextran and glucan contents in doughs fermented by *Weissella* spp. compared with *Leuconostoc* spp.,
286 since *Weissella* spp. preferentially produced only low-branched dextran while *Leuconostoc* spp. tend
287 to produce more than one type of dextran (Cote and Skory, 2015; Maina et al., 2008).

288 After 24 h fermentation, mannitol was detected in all doughs except doughs fermented by *W. confusa*
289 Sj5-4 (Sj5-4_CT and Sj5-4_S) (Table 4). Mannitol content varied from 0.02% to 0.46% in control
290 doughs and from 0.21% to 4.22% in sucrose-enriched doughs, with a strong inducing effect due to
291 sucrose addition. The highest mannitol content was found in 5577_S, corresponding to its highest
292 acetic acid content (Table 2) since the reduction of fructose could induce acetic acid formation
293 (Wisselink et al., 2002). By contrast, the lowest mannitol contents in control and sucrose-enriched
294 doughs were found in 15878_CT and 15878_S. Generally, *Leuconostoc* spp. produce mannitol from
295 fructose by the activity of mannitol dehydrogenase, while *Weissella* spp. ferment fructose but do not
296 reduce it to mannitol, leading to decreased or no mannitol production (Erten, 1998; Galle et al., 2010).
297 However, under the conditions of our study, *W. cibaria* Sj 1b produced a higher amount of mannitol
298 than did *Ln. pseudomesenteroides* DSM 20193 which was previously reported as a good mannitol
299 producer when grown in the presence of fructose or sucrose (Vandamme et al., 1987), indicating that
300 mannitol production was both strain- and substrate-dependent (Galle et al., 2010).

301 **3.4 Sucrose utilization**

302 It was previously shown that during EPS synthesis, sucrose was first utilized for microbial growth
303 during the exponential phase, and then for dextran production during the stationary phase, liberating

304 fructose (Han et al., 2014; Plante and Shriver, 1998). The liberated fructose was further reduced to
305 mannitol in *Leuconostoc* spp. by mannitol dehydrogenase, while in *Weissella* spp., the liberated
306 fructose was fermented but not reduced to mannitol (Galle et al., 2010). Theoretically, the total
307 content of residual fructose and mannitol should be roughly equal to the content of glucan formed.
308 However, due to the usage of sucrose-liberated glucose for the growth of LAB and other
309 microorganisms (potentially present in fava bean flour), the production of acids and other volatile
310 compounds and the formation of oligosaccharides by glucansucrase through acceptor reaction, the
311 content of glucan was generally lower than the total content of residual fructose and mannitol (Tables
312 3 and 4). Actually, in a complex system such as any food matrix, the sugar balance is difficult to
313 achieve, as also reported by other researchers who used carrot puree as the medium to produce EPS
314 (Juvonen et al., 2015). Furthermore, significant differences were found in residual fructose and
315 mannitol levels between sucrose-enriched doughs fermented by different LAB strains. The total
316 content of residual fructose and mannitol in sucrose-enriched doughs fermented by *Leuconostoc* spp.
317 (20193_S, 20240_S and 5577_S) was generally in line with the theoretical amount of fructose
318 released from the original and the added sucrose. However, in sucrose-enriched doughs fermented by
319 *Weissella* spp. (15878_S and Sj 1b_S), the residual fructose and mannitol content were lower than the
320 theoretical maximal fructose content. It could be hypothesized that fructose was preferable to glucose
321 for *Weissella* spp. since different strains might have different carbon source preferences (Kajala et al.,
322 2015).

323 **3.5 Rheological properties**

324 **3.5.1 Viscosity change and WHC**

325 According to the viscosity flow curves, showing in the supplementary material (Fig. S1 and S2), all
326 doughs showed a typical shear-thinning behavior after fermentation. The initial viscosity values of
327 sucrose-enriched doughs were lower than control doughs due to their lower flour content (Table 5).
328 After fermentation, the viscosity of all doughs increased to different extents, indicating the thickening

329 ability of EPS. In detail, the viscosity in control doughs ranged from 1.73 to 2.78 Pa·s before
330 fermentation and from 2.59 to 5.87 Pa·s after fermentation, increasing by up to 3.28 Pa·s. The highest
331 viscosity increase was found in Sj 1b_CT, corresponding to its highest dextran content among control
332 doughs (Table 4). In sucrose-enriched doughs, the viscosity ranged from 0.20 to 0.35 Pa·s before
333 fermentation and from 0.82 to 14.77 after fermentation, increasing by up to 14.57 Pa·s. The highest
334 viscosity increase was found in 20193_S, corresponding to its highest dextran content as well (Table
335 4). Interestingly, doughs fermented by *W. confusa* Sj5-4 (Sj5-4_CT and Sj5-4_S) showed the lowest
336 viscosity after fermentation although their dextran contents were not the lowest. This low viscosity
337 improving effect might be due to the structure and molecular weight of dextran produced by *W.*
338 *confusa* Sj5-4, which might influence its rheological behavior; however, more evidences are needed
339 to prove this hypothesis.

340 The WHC of food has been defined as the ability to hold its own and added water during the
341 application of forces, pressing, centrifugation or heating (Zayas, 1997). This is a limiting factor in
342 protein food applications (Zayas, 1997) and is also an important characteristic for evaluating the
343 function of EPS as food hydrocolloids. Among all doughs, 20193_S showed the highest WHC value
344 with significant difference, while the dough fermented by *L. plantarum* DPPMAB24W (B24W_S)
345 showed the lowest value, as it was an EPS-negative control (Table 6). In other sucrose-enriched
346 doughs, WHC values ranged from 83.72% to 89.26%, without much difference.

347 **3.5.2 Hysteresis loop**

348 A hysteresis loop was frequently observed in viscoelastic materials during the shear rate sweep, and
349 was assumed to be the difference between the energy required for structural breakdown and
350 rebuilding (Gambuś et al., 2004). Materials with larger hysteresis loop areas tend to have better
351 structural reversibility (Purwandari et al., 2007). In our study, the hysteresis loops of sucrose-enriched
352 doughs fermented by different LAB strains varied considerably (Fig. 2). As expected, 20193_S
353 showed the largest loop due to its highest dextran content, while the EPS negative control B24W_S

showed the smallest loop. In order to get specific loop areas, the loop area of each dough was calculated and is presented in Table 6. Consistent with previous data, the largest loop area (7.98 units) was observed in 20193_S, indicating its best structural reversibility among all doughs. In contrast, B24W_S showed the worst structural reversibility as there was no texture modification from EPS in this dough. No significant difference was found between Sj5-4_S and B24W_S in loop area, indicating a weak texture modification of dextran produced by *W. confusa* Sj5-4. Generally, sucrose-enriched doughs fermented by *Leuconostoc* spp. showed a better structural reversibility than those fermented by *Weissella* spp., except Sj 1b_S, which showed a comparable structural reversibility with 20240_S and 5577_S (Table 6).

3.5.3 Elastic modulus

The elastic modulus (G') increased with the rising frequency in all sucrose-enriched doughs (Fig. 3). In particular, 20193_S and Sj 1b_S showed the highest G' at any frequency, indicating more elastic and solid characters compared with others. Correspondingly, as an EPS-negative control, B24W_S showed the lowest G' at any frequency. It was previously reported that the dependence of the elastic modulus with frequency gives information about the gel structure (Stading and Hermansson, 1990). According to Fig. 3, 20193_S and Sj 1b_S were less frequency-dependent compared with other doughs, indicating stable and different gel structures formed in these two doughs. The G' values of all sucrose-enriched doughs at the frequency of 1.0 Hz were compared, showing a considerable variation ranging from 33.85 to 1656.03 Pa (Table 6). With EPS synthesized *in situ*, the G' values of the doughs were significantly higher than the EPS-negative control (B24W_S), revealing the function of EPS in gel structure strengthening. Outstandingly, although the dextran content of Sj 1b_S was about half that of 20193_S, they both had G' values over 1500 Pa, indicating a strong network between EPS and fava bean proteins in these two doughs. It was reported that the interaction between EPS and proteins was driven by the chemical structure of EPS (Folkenberg et al., 2006). Therefore, more studies have to be performed on chemical structures, molecular weight distributions and rheological

behaviors of dextrans produced by *Ln. pseudomesenteroides* DSM 20193 and *W. cibaria* Sj 1b. The dynamic oscillatory sweep curves are presented in the supplementary material (Fig. S3), and all sucrose-enriched doughs showed a solid-like behavior, except 20240_S.

Currently, the rheological properties of yogurt containing EPS and the network between EPS and milk proteins have been extensively studied (Amatayakul et al., 2006; Gentès et al., 2013; Purwandari et al., 2007). However, little is known about the influence of EPS on the rheological properties of legume-made doughs and the network between EPS and legume proteins. Therefore, further research is still needed.

To conclude, fava bean doughs fermented by different LAB strains showed different chemical and rheological properties. The addition of sucrose facilitated EPS formation, which further affected the rheological properties of fermented doughs. It has been previously observed that the viscosifying efficiency of EPS also depends on their physical-chemical properties, highlighting the importance of certain structure-function relationships for specific technological applications (Ruas-Madiedo et al., 2002). As a consequence, this also emphasizes the role of selected LAB to produce specific EPS that will behave in a desired way when incorporated into food products. In this work, *Ln. pseudomesenteroides* DSM 20193 showed the highest potential in EPS production and texture modification in fava bean dough. The fermented doughs with synthesized *in situ* EPS may meet consumers' needs for "clean labels" and may have a potential use in the food industry. Further studies should be performed on the application of fermented legumes in different food products.

Supplementary material

The viscosity flow curves of control and sucrose-enriched fava bean doughs fermented by *Ln. pseudomesenteroides* DSM 20193, *Ln. mesenteroides* DSM 20240, *Ln. citreum* DSM 5577, *W. cibaria* DSM 15878, *W. cibaria* Sj 1b and *W. confusa* Sj5-4 before and after fermentation (Fig. S1 and Fig. S2). The dynamic oscillatory sweep curves of sucrose-enriched fava bean doughs fermented

by *Ln. pseudomesenteroides* DSM 20193, *Ln. mesenteroides* DSM 20240, *Ln. citreum* DSM 5577, *W. cibaria* DSM 15878, *W. cibaria* Sj 1b, *W. confusa* Sj5-4 and *L. plantarum* DPPMAB24W after fermentation (Fig. S3).

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Figure captions

Fig. 1. HPAEC-PAD chromatograms of control doughs made of native or autoclaved fava bean flour fermented by *Leuconostoc* spp. (A) and *Weissella* spp. (B). The chromatogram of unfermented control dough without starter is also presented (CT_0h). Other chromatograms are: *Ln. pseudomesenteroides* DSM 20193 fermented control dough made of native (20193_N) or autoclaved flour (20193_A); *Ln. mesenteroides* DSM 20240 fermented control dough made of native (20240_N) or autoclaved flour (20240_A). The rest of the chromatograms continue like this in the order of *Ln. citreum* DSM 5577, *W. cibaria* DSM 15878, *W. cibaria* Sj 1b and *W. confusa* Sj5-4 (Gal, galactose; Suc, sucrose; Raf, raffinose; Sta, stachyose; Ver, verbascose).

Fig. 2. Hysteresis loops of sucrose-enriched fava bean doughs fermented by *Ln. pseudomesenteroides* DSM 20193 (20193_S), *Ln. mesenteroides* DSM 20240 (20240_S), *Ln. citreum* DSM 5577 (5577_S), *W. cibaria* DSM 15878 (15878_S), *W. cibaria* Sj 1b (Sj 1b_S), *W. confusa* Sj5-4 (Sj5-4_S) and *L. plantarum* DPPMAB24W (B24W_S).

Fig. 3. Elastic modulus (G') of sucrose-enriched fava bean doughs fermented by *Ln. pseudomesenteroides* DSM 20193 (20193_S), *Ln. mesenteroides* DSM 20240 (20240_S), *Ln. citreum* DSM 5577 (5577_S), *W. cibaria* DSM 15878 (15878_S), *W. cibaria* Sj 1b (Sj 1b_S), *W. confusa* Sj5-4 (Sj5-4_S) and *L. plantarum* DPPMAB24W (B24W_S) as a function of oscillatory frequency (0.1 – 10 Hz).

551 Tables

552 Table 1. Sample codes and ingredients of control and sucrose-enriched fava bean doughs fermented
553 by *Leuconostoc* spp., *Weissella* spp. and *Lactobacillus plantarum* DPPMAB24W.

Sample code	Flour (g)	Sucrose (g)	Water (mL)	Starter
20193_CT [a]	40	0	60	*Leuconostoc pseudomesenteroides* DSM 20193
20193_S [b]	30	10	60	
20240_CT	40	0	60	*Leuconostoc mesenteroides subsp. mesenteroides* DSM 20240
20240_S	30	10	60	
5577_CT	40	0	60	*Leuconostoc citreum* DSM 5577
5577_S	30	10	60	
15878_CT	40	0	60	*Weissella cibaria* DSM 15878
15878_S	30	10	60	
Sj 1b_CT	40	0	60	*Weissella cibaria* Sj 1b
Sj 1b_S	30	10	60	
Sj5-4_CT	40	0	60	*Weissella confusa* Sj5-4
Sj5-4_S	30	10	60	
B24W_S	30	10	60	*Lactobacillus plantarum* DPPMAB24W

554 [a] CT means control dough. [b] S means sucrose-enriched dough.

555 Table 2. LAB growth and acidification of control and sucrose-enriched fava bean doughs fermented by *Leuconostoc* spp. and *Weissella* spp.. The
556 table shows cell density, cell density increase, pH, TTA, citric acid, lactic acid and acetic acid concentrations together with their ratios (FQ).

Sample code [A]	Cell density [D] (log cfu/g)	Δ log [E]	pH	TTA (mL)	Citric acid (mmol/100 g dough)	Lactic acid	Acetic acid	FQ [F]
CT_0h [B]	-	-	6.6 ± 0.00	4.0 ± 0.05	2.29 ± 0.05	nd	nd	-
S_0h [C]	-	-	6.7 ± 0.01	2.9 ± 0.03	1.80 ± 0.02	nd	nd	-
20193_CT	9.7 ± 0.2 $^{a, c}$	3.2 ± 0.2 $^{a, d}$	4.4 ± 0.00 ^{a}	17.2 ± 0.11 ^{a}	2.69 ± 0.10 ^{a}	21.97 ± 0.91 ^{a}	6.46 ± 0.37 $^{a, d}$	3.40
20193_S	9.8 ± 0.1 ^{a}	3.4 ± 0.1 ^{a}	4.3 ± 0.00 ^{b}	15.6 ± 1.27 $^{a, b, e}$	2.00 ± 0.07 $^{a, b}$	17.63 ± 0.96 $^{b, e}$	7.46 ± 0.28 $^{a, b, d}$	2.36
20240_CT	8.9 ± 0.0 $^{b, d}$	3.1 ± 0.0 $^{a, b, d}$	4.8 ± 0.00 ^{c}	14.5 ± 0.04 $^{b, c, e}$	1.39 ± 0.26 ^{b}	13.96 ± 0.59 $^{b, d}$	6.11 ± 0.31 ^{a}	2.28
20240_S	8.6 ± 0.1 ^{b}	2.8 ± 0.1 $^{b, d}$	4.5 ± 0.01 ^{d}	15.7 ± 0.05 $^{a, b}$	1.21 ± 0.12 ^{b}	9.50 ± 0.32 ^{c}	9.34 ± 0.45 $^{b, d}$	1.02
5577_CT	9.5 ± 0.0 ^{c}	3.9 ± 0.0 ^{c}	4.9 ± 0.02 ^{e}	13.3 ± 0.14 $^{c, e, f}$	nd	14.08 ± 0.60 $^{b, d}$	9.44 ± 0.42 $^{b, d}$	1.49
5577_S	9.6 ± 0.0 $^{a, c}$	4.0 ± 0.1 ^{c}	4.3 ± 0.00 ^{b}	19.8 ± 0.24 ^{d,}	nd	12.45 ± 0.59 $^{c, d, f}$	16.24 ± 1.28 ^{c}	0.77
15878_CT	9.0 ± 0.1 ^{d}	2.9 ± 0.1 ^{d}	4.8 ± 0.01 ^{c}	15.0 ± 0.07 $^{b, e,}$	1.73 ± 0.38 $^{a, b}$	17.47 ± 1.69 $^{b, e}$	7.63 ± 0.57 $^{a, b, d}$	2.29
15878_S	9.0 ± 0.0 ^{d}	2.9 ± 0.1 ^{d}	4.6 ± 0.04 ^{f}	14.4 ± 0.58 $^{b, c, e}$	1.26 ± 0.35 ^{b}	15.98 ± 0.99 $^{b, d, e}$	6.56 ± 0.72 $^{a, d}$	2.43
Sj 1b_CT	9.0 ± 0.0 ^{d}	3.2 ± 0.1 $^{a, d}$	4.8 ± 0.02 ^{c}	14.7 ± 0.33 $^{b, c, e}$	0.94 ± 0.59 ^{b}	14.57 ± 0.73 ^{b}	7.37 ± 0.30 $^{a, b, d}$	1.98
Sj 1b_S	9.0 ± 0.0 ^{d}	3.3 ± 0.1 ^{a}	4.6 ± 0.04 ^{f}	14.0 ±0.52 $^{e, f}$	1.22 ± 0.31 ^{b}	9.64 ± 0.26 ^{c}	7.62 ± 0.58 $^{a, b, d}$	1.26
Sj5-4_CT	9.0 ± 0.1 ^{d}	3.1 ± 0.1 $^{a, b, d}$	5.0 ± 0.01 ^{e}	12.6 ± 0.13 ^{f}	nd	18.41 ± 2.41 $^{a, e}$	8.65 ± 1.46 ^{d}	2.13
Sj5-4_S	9.1 ± 0.1 ^{d}	3.2 ± 0.1 $^{a, b, d}$	4.8 ± 0.01 ^{c}	10.7 ± 0.11 ^{g}	nd	14.78 ± 0.42 $^{b, f}$	6.55 ± 0.31 $^{a, d}$	2.26

557 [A] Details about the sample code can be found in Table 1. [B] CT_0h, control dough without starters before fermentation. [C] S_0h, sucrose-enriched
558 dough without starters before fermentation. [D] LAB cell density after fermentation. [E] The increase of LAB cell density after fermentation. [F] FQ,
559 fermentation quotient. [a-g] Values in the same column with different letters are significantly different ($p < 0.05$).

560 Table 3. Mono-, di- and oligo-saccharides in control and sucrose-enriched fava bean doughs fermented by *Leuconostoc* spp. and *Weissella* spp.
561 before and after fermentation.

Sample code [a]	Sugars [d] (%, w/w, wet matter)						
	Glc (%)	Suc (%)	Fru (%)	Gal (%)	Raf (%)	Sta (%)	Ver (%)
CT_0h [b]	nd [e]	1.33 ± 0.07	nd	nd	0.12 ± 0.01	0.91 ± 0.01	1.94 ± 0.02
S_0h [c]	nd	11.65± 0.05	nd	nd	0.09 ± 0.01	0.68 ± 0.01	1.45 ± 0.01
20193_CT	nd	nd	nd	0.20 ± 0.01	nd	0.24 ± 0.00	0.31 ± 0.03
20193_S	nd	nd	5.73 ± 0.10	0.34 ± 0.01	nd	nd	nd
20240_CT	nd	nd	nd	0.44 ± 0.01	nd	0.58 ± 0.01	0.78 ± 0.04
20240_S	0.36 ± 0.02	0.30 ± 0.05	3.30 ± 0.02	0.34 ± 0.00	nd	nd	nd
5577_CT	nd	nd	nd	0.56 ± 0.01	nd	0.85 ± 0.05	0.99 ± 0.02
5577_S	nd	nd	2.06 ± 0.10	0.45 ± 0.02	nd	nd	nd
15878_CT	nd	nd	nd	0.48 ± 0.01	nd	0.63 ± 0.01	0.85 ± 0.01
15878_S	nd	5.77 ± 0.37	1.76 ± 0.21	0.34 ± 0.00	nd	nd	nd
Sj 1b_CT	nd	nd	nd	0.31 ± 0.01	nd	0.85 ± 0.03	0.84 ± 0.03
Sj 1b_S	nd	4.27 ± 0.48	2.19 ± 0.07	0.31 ± 0.00	nd	nd	nd
Sj5-4_CT	nd	nd	nd	0.30 ± 0.00	nd	0.54 ± 0.00	0.83 ± 0.02
Sj5-4_S	nd	nd	5.37 ± 0.03	0.30 ± 0.01	nd	nd	nd

562 [a] Details about the sample code can be found in Table 1. [b] CT_0h, control dough without starters before fermentation. [c] S_0h, sucrose-enriched
563 dough without starters before fermentation.[d] Sugars: Glc, glucose; Suc, sucrose; Fru, fructose; Gal, galactose; Raf, raffinose; Sta, stachyose; Ver,
564 verbascose. [e] nd = not detected.

565 Table 4. The contents of dextran, glucan and mannitol in control and sucrose-enriched fava bean
566 doughs fermented by *Leuconostoc* spp. and *Weissella* spp. [A]

Sample code [B]	Dextran (%)	Glucan (%)	Mannitol (%)
20193_CT	0.21 ± 0.09 [a, e]	0.32 ± 0.01 [a]	0.16 ± 0.01 [a, c]
20193_S	3.67 ± 0.07 [b]	4.15 ± 0.13 [b]	0.27 ± 0.03 [a, c]
20240_CT	0.42 ± 0.01 [a, e]	0.49 ± 0.37 [a]	0.46 ± 0.01 [a, c]
20240_S	2.85 ± 0.11 [c]	4.33 ± 0.30 [b]	2.01 ± 0.98 [a]
5577_CT	0.11 ± 0.04 [a]	0.53 ± 0.25 [a]	0.28 ± 0.00 [a, c]
5577_S	2.17 ± 0.03 [d]	3.77 ± 0.11 [b, c]	4.22 ± 1.31 [b]
15878_CT	0.46 ± 0.04 [e]	0.50 ± 0.02 [a]	0.02 ± 0.00 [c]
15878_S	2.11 ± 0.11 [d, f]	2.71 ± 0.48 [c]	0.21 ± 0.10 [a, c]
Sj 1b_CT	0.74 ± 0.11 [e]	0.82 ± 0.03 [a]	0.34 ± 0.01 [a, c]
Sj 1b_S	1.86 ± 0.09 [d]	2.66 ± 0.76 [c]	1.39 ± 0.31 [a, c]
Sj5-4_CT	0.32 ± 0.08 [a, e]	0.32 ± 0.13 [a]	nd [C]
Sj5-4_S	2.24 ± 0.13 [f]	2.57 ± 0.28 [c]	nd

567 [A] The contents were calculated on wet weight base. [B] Details about the sample code can be found in
568 Table 1. [C] nd = not detected. [a-f] Values in the same column with different letters are significantly
569 different ($p < 0.05$).

570 Table 5. Initial, final viscosity and viscosity increase of control and sucrose-enriched fava bean
571 doughs fermented by *Leuconostoc* spp. and *Weissella* spp.. [A]

Sample code [B]	Initial viscosity (Pa·s)	Final viscosity (Pa·s)	Viscosity increase (Pa·s)
Control dough			
20193_CT	1.73 ± 0.20 [a]	3.16 ± 0.20 [a]	1.43
20240_CT	2.78 ± 0.20 [b]	4.57 ± 0.10 [b]	1.79
5577_CT	2.54 ± 0.20 [b]	3.92 ± 0.30 [b]	1.38
15878_CT	2.62 ± 0.00 [b]	4.27 ± 0.20 [b]	1.65
Sj 1b_CT	2.59 ± 0.10 [b]	5.87 ± 0.30 [c]	3.28
Sj5-4_CT	2.59 ± 0.10 [b]	2.59 ± 0.10 [a]	0.00
Sucrose-enriched dough			
20193_S	0.20 ± 0.00 [a]	14.77 ± 0.00 [a]	14.57
20240_S	0.35 ± 0.00 [b]	6.16 ± 0.10 [b]	5.81
5577_S	0.28 ± 0.00 [c, e]	6.24 ± 0.40 [b]	5.96
15878_S	0.33 ± 0.00 [b, d]	4.28 ± 0.60 [c]	3.95
Sj 1b_S	0.31 ± 0.00 [c, d]	6.54 ± 0.20 [b]	6.23
Sj5-4_S	0.27 ± 0.00 [e]	0.82 ± 0.00 [d]	0.55

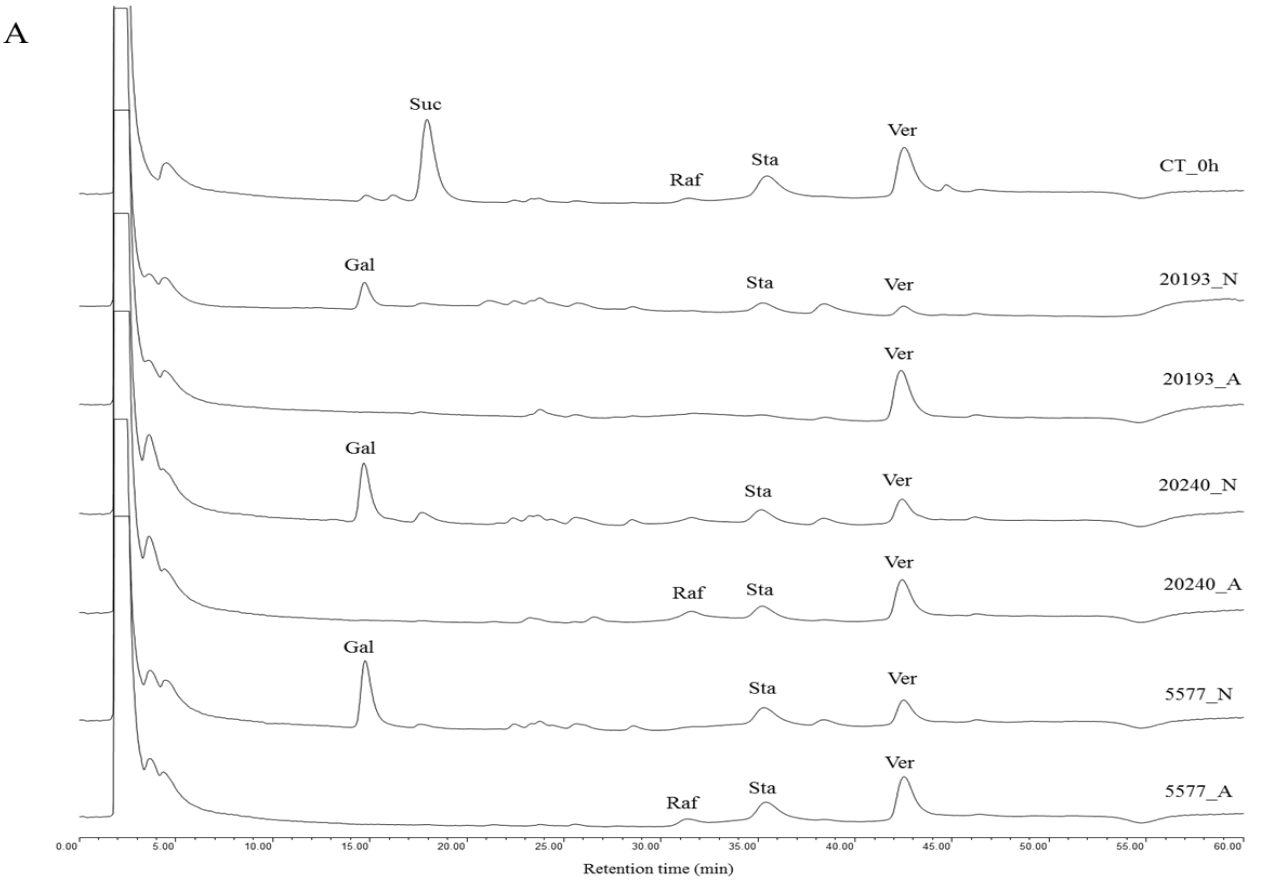
572 [A] The viscosity values of all samples were taken at the shear rate of 100 1/s. [B] Details about the sample
573 code can be found in Table 1. [a-e] Values in the same column with different letters are significantly
574 different ($p < 0.05$).

575 Table 6. Water holding capacity (WHC), hysteresis loop area and elastic modulus (G') of sucrose-
576 enriched fava bean doughs fermented by *Leuconostoc* spp., *Weissella* spp. and *Lactobacillus*
577 *plantarum* B24W.

Sample code [A]	WHC (%, w/w)	Hysteresis loop area (Unit) [B]	G' [C] (Pa)
20193_S	99.16 ± 0.33 [a]	7.98 ± 0.63 [a]	1588.28 ± 58.16 [a]
20240_S	88.33 ± 1.65 [b]	2.82 ± 0.01 [b]	339.42 ± 29.17 [b, d]
5577_S	86.41 ± 1.43 [b, c]	2.14 ± 0.02 [b, c]	667.77 ± 65.49 [c]
15878_S	87.77 ± 1.26 [b, c]	1.40 ± 0.04 [c]	539.92 ± 76.84 [b, c]
Sj 1b_S	89.26 ± 1.51 [b]	2.52 ± 0.22 [b]	1656.03 ± 93.94 [a]
Sj5-4_S	83.72 ± 1.37 [c, d]	0.28 ± 0.01 [d]	159.70 ± 105.35 [d, e]
B24W_S	81.00 ± 0.59 [d]	0.14 ± 0.01 [d]	33.85 ± 13.87 [e]

578 [A] Details about the sample code can be found in Table 1. [B] Unit = 10^6 Pa/s. [C] G' values were taken at
579 the frequency of 1.0 Hz. [a-e] Values in the same column with different letters are significantly different
580 ($p < 0.05$).

581 Fig. 1



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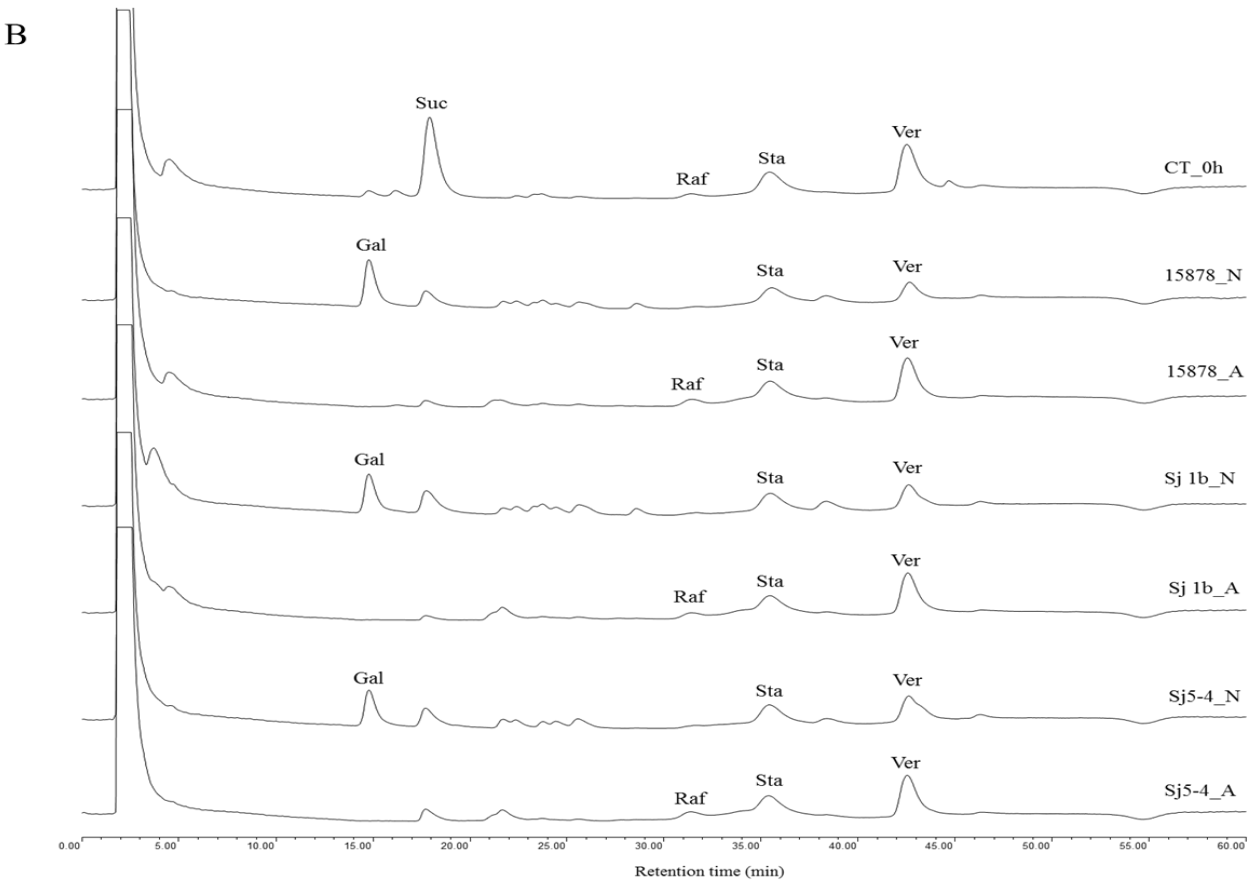
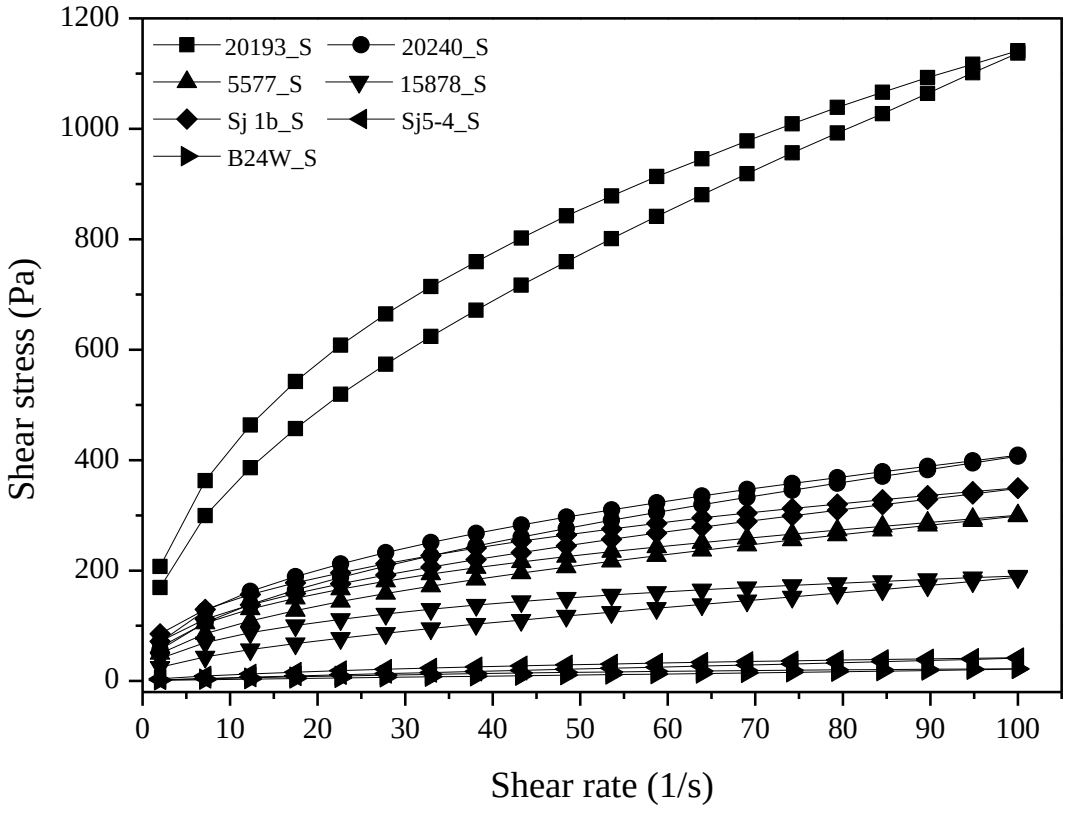


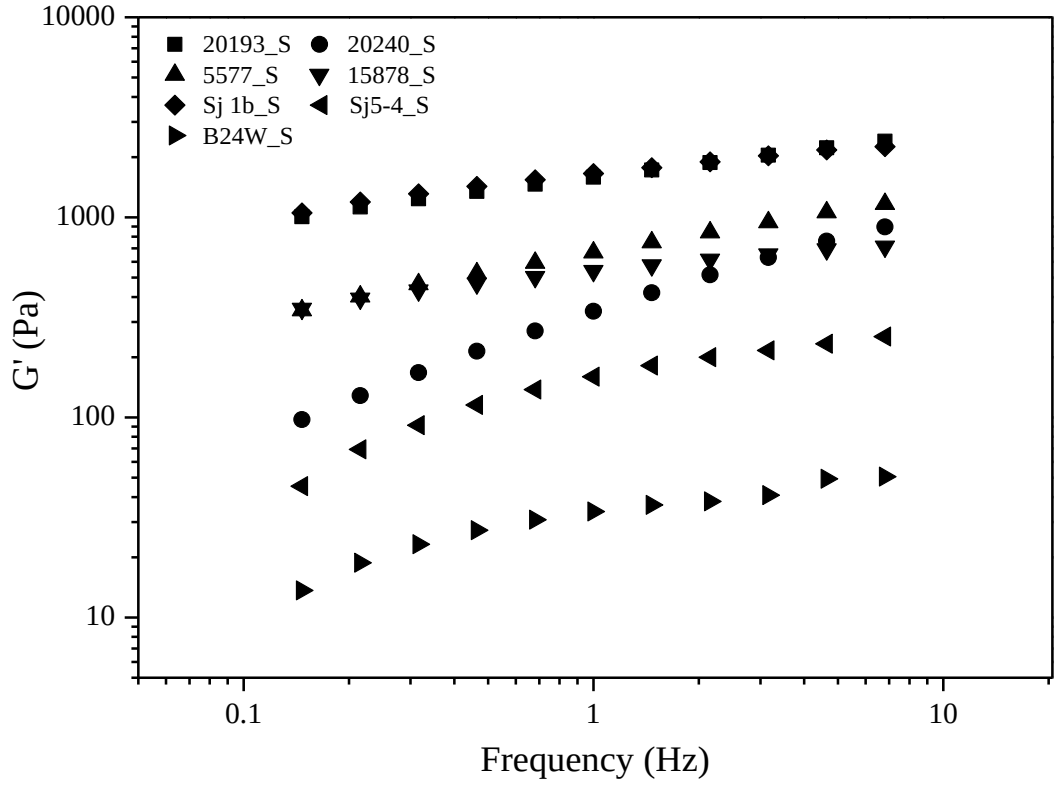
583

584 Fig. 2



585

586 Fig. 3



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